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13. ABSTRACT (Maximum 200 Words)

Because of the potential synergistic interaction between an anti-angiogenic aminosterol, squalamine, and other angiogenic modifiers such as vascular endothelial growth factor (VEGF) and cytokines that may be released during intermittent androgen withdrawal therapy, we tested extensiviely the interaction between squalamine and VEGF for an enhanced cytotoxicity to human prostate cancer cells in vitro and xenografts tumor models in vivo. While in vitro synergistic interaction was demonstrated specifically in human prostate cancer cell lines containing a functional androgen receptor, we encountered difficulty in demonstrating such synergism in vivo for the reason that severe toxicity was noted when VEGF was delivered as an Ad-CMV-TK vector. For this reason, we explored the other possible synergistic interaction between squalamine and castration. Results and Discussion: Squalamine is highly synergistic to castration-induced endothelial destruction when applied at the time of castration. We noted VEGF receptor, flt-1 and integrin profile (e.g. $\alpha6\beta4$) can predict squalamine response. Prostate cancer cells lacking the expression of these markers may be less responsive to the synergistic ineraction between squalamine and castration. We are currently assessing the possible interaction between squalamine and VEGF and squalamine and androgen status of the cell culture and in animals subjected to castration to evaluate if synergism may exist particularly against the growth of endothelial cells.

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Introduction:

The objective of this proposal is to seek for a combination therapy between a low molecular weight aminosterol squalamine which has anti-angiogenic activity against induced endothelial proliferation and migration and vascular endothelial growth factor, VEGF, on the growth of human prostate tumors both *in vitro* and *in vivo*. Although both agents when applied alone have little anti-tumor effect, they have remarkable synergistic action when applied together in tumor cells that express certain profiles of integrin isotypes and VEGF receptors. This approach is taken because of the known inherent genetic stability of endothelial cells which are required for tumor cells' continued growth and expansion and the potential clinical application of an effective combination therapy targeted at tumor and its endothelial supplies for the effective treatment of hormone refractory prostate cancers.

Body:

Task 1: Establishment of in vivo human prostate tumors:

This task has been completed. Please see previous progress report.

Task 2: Construction, characterization and production of adenoviruses that contain VEGF driven by a CMV universal promoter:

This task has been completed. Please see previous progress report.

Task 3: Evaluation of the *in vitro* and *in vivo* synergism between squalamine and VEGF (or castration), and assessment of the biochemical and morphologic changes of the prostatic tissues in vivo:

This task has been completed. Please see previous progress report.

Task 4: Determine the *in vitro* effect of squalamine and/or VEGF on the growth of prostatic and endothelial cells:

The effect of squalamine and/or VEGF on the growth of prostatic cancer epithelial cells has been presented in our previous progress report. We have obtained the growth inhibitory effect of squalamine on human endothelial cell line HUVEC. In the descending order of sensitivity to squalamine were: HUVEC appears to be more sensitive, LNCaP/C4-2 was intermediate and PC3/PC3M/DU145 were least sensitive (Figure 1 and 2). Unlike prostate cancer epithelial cells, no growth inhibitory synergism was noted between VEGF and squalamine on HUVEC cells nor this inhibition was affected by $\alpha v\beta_3$ antibody, suggesting ECM-integrin mediated effects may not be responsible for squalamine-mediated cytotoxicity (Figure 2). In addition, we also found that squalamine exerted marked effect in inhibiting the migration of HUVEC cells on plastic and morphogenesis (e.g. tubular formation) *in vitro* (Figure 3). Activity of

squalamine and/or VEGF on the growth of endothelial cells are being evaluated. Results of squalamine on tumor and endothelial growth, migration and tubular formation is described in a prepared abstract (Appendix A).

Task 5: Recording of the morphologic changes of cells after squalamine and/or VEGF treatment:

This task has been completed. Please see previous progress report.

Task 6: Evaluation of the relationship between morphologic changes of prostate cancer and endothelial cells *in vitro* after squalamine and/or VEGF treatment with that of their biochemical expression of TSP-1 and cell surface integrin isotypes:

In a previous progress report, we have completed the analysis of morphologic and biochemical features of prostate tumors after squalamine treatment in intact and castrated hosts. We are presently conducting similar analysis in chimeric tissue culture models consisting of prostate cancer cells, HUVEC cells and prostate or bone stroma cells as a 3D culture. Figure 4 shows three different color-coated cells, epithelial, stromal and endothelial cells, when viewed under a confocal microscope. This new model system will be used for the evaluation of the biochemical expression of TSP-1 and the cell surface integrin isotypes and other relevant gene expressions in the presence or absence of squalamine and/or VEGF treatment.

Task 7: Confirmation of the above biochemical responses of prostate cancer cells and endothelial cells to squalamine and VEGF *in vivo*.

We have established HUVEC cell culture *in vitro* and found that HUVEC cells are exquisitely sensitive to squalamine. The combined effect of squalamine and VEGF will be evaluated in the chimeric tumor model presented in Task 6 above.

Task 8: Evaluation of methodologies for evaluating signal cascade and apoptosis following VEGF and squalamine.

The effect of VEGF and squalamine on focal adhesion kinase (PP125FAK) and its closely related Pyk2 are currently under investigation. We have, however, established the basic conditions, such as extracellular matrixes, growth factors and androgen conditions that may be needed for the proper expression of FAK and Pyk2 and status of phosphorylation.

Task 9: Evaluation of changes in signal transduction components following exposure to squalamine and/or VEGF *in vitro* and confirmation of such changes in prostate tumor models *in vivo*.

This task is currently under investigation.

Task 10: Characterization of changes of signal transduction components and their relationship to apoptosis, and comparison of their activity both *in vivo* and *in vitro*.

This task is also under investigation.

Key Research Accomplishments:

- We have established HUVEC endothelial culture *in vitro* and have determined the effect of squalamine on this cell line. We found that the sensitivity toward squalamine in the following descending order HUVEC more sensitive, LNCaP/C4-2 intermediate sensitive and PC3/DU145 least sensitive.
- We have established a color-coded chimeric culture of prostatic cancer cells with HUVEC and prostatic or bone stromal cells. These prostatic organoids can be visualized under confocal microscope. We have established methods to measure both the basal and the phosphorylation assay for FAK and Pyk2.

Reportable Outcomes:

1. We have written an abstract to be submitted to the American Society for Cancer Research.
2. A review article on tumor stroma interaction is published in *Differentiation* 70:506-521, 2002.

Conclusions:

VEGF and squalamine synergism appears to be a phenomenon *in vitro* and its *in vivo* synergism is more difficult to demonstrate due to severe toxicity of delivery of VEGF to tumor tissues in tumor-bearing animals. The concept to enhance tumor and endothelial cell death using angiogenic modifiers however received support by the application of squalamine immediately after castration. Based on immunohistochemical data, it appears that tumor cells overexpress VEGF receptor, flt-1 and specific integrin isotype, such as $\alpha6\beta4$, are responders. This part of the work is currently pursued in Dr. Mitch Sokoloff's lab with addition of radiation and squalamine as a new combination. This work will be further explored and will be the subject of a future human clinical trial.

References:

None

Appendix:

1. Jin F, Sokoloff MH, Sen B, Ling J, Hsieh CL, Zhau EK and Chung LWK. Development of Squalamine as an Anti-angiogenic Agent for the Treatment of

Human Prostate Cancer in Experimental Models. Abstract for American Society for Cancer Research.

2. Sung SY and Chung LWK. Prostate tumor-stroma interaction: molecular mechanisms and opportunities for therapeutic targeting. *Journal of Differentiation 70:506-521, 2002.*

Effect of Squlamine on the Growth of Human Prostate Cancer Cells

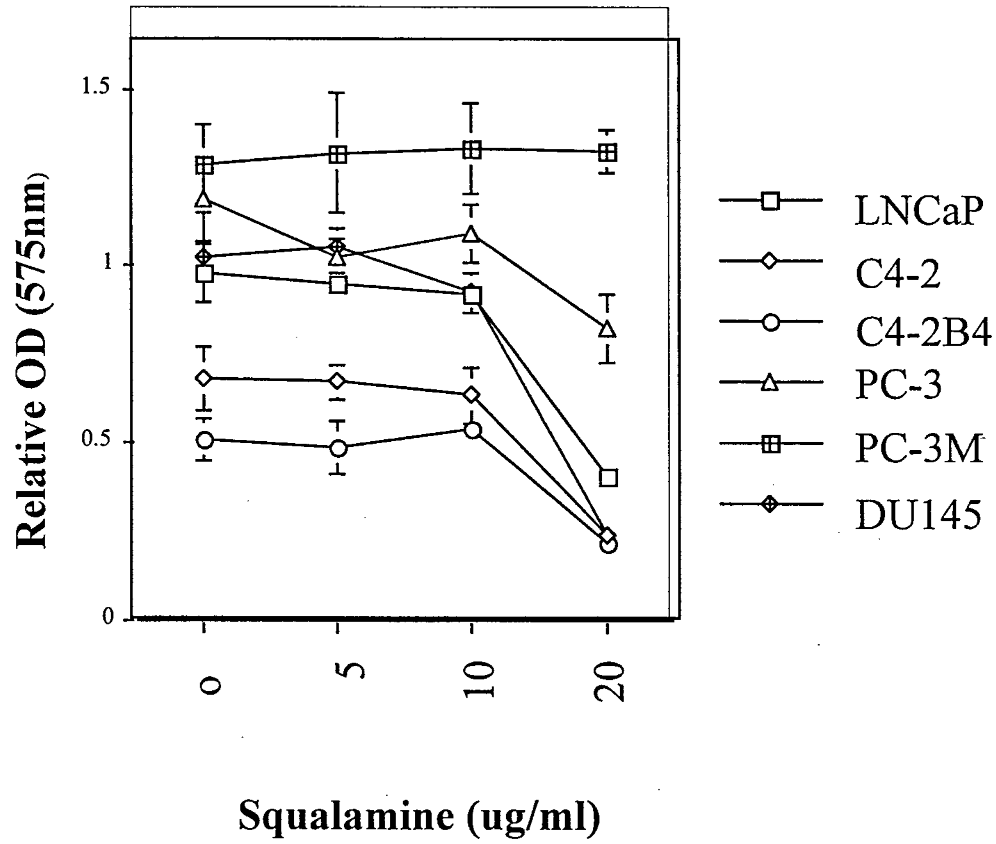

Figure 1

Effects of Squalamine (SQ), Vascular Endothelial Cell Growth Factor (VEGF) and/or $a_v \beta_3$ Integrin Antibody, LM609, on the Growth of HUVEC Cells In Vitro

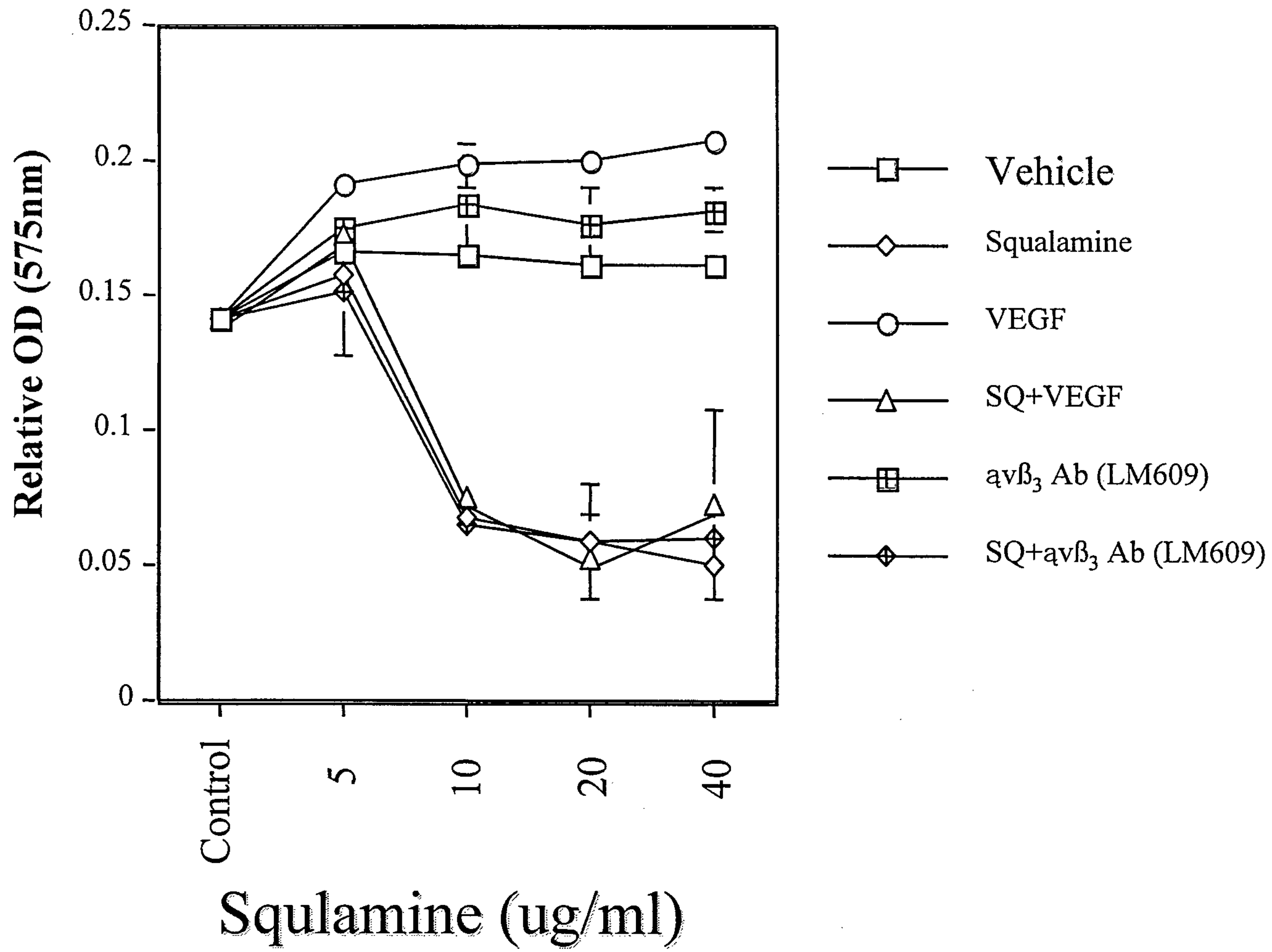

Figure 2

Effects of SQ and/or $à_v ß_3$ Atibody LM609 on the Tubular Formation of HUVEC Cells

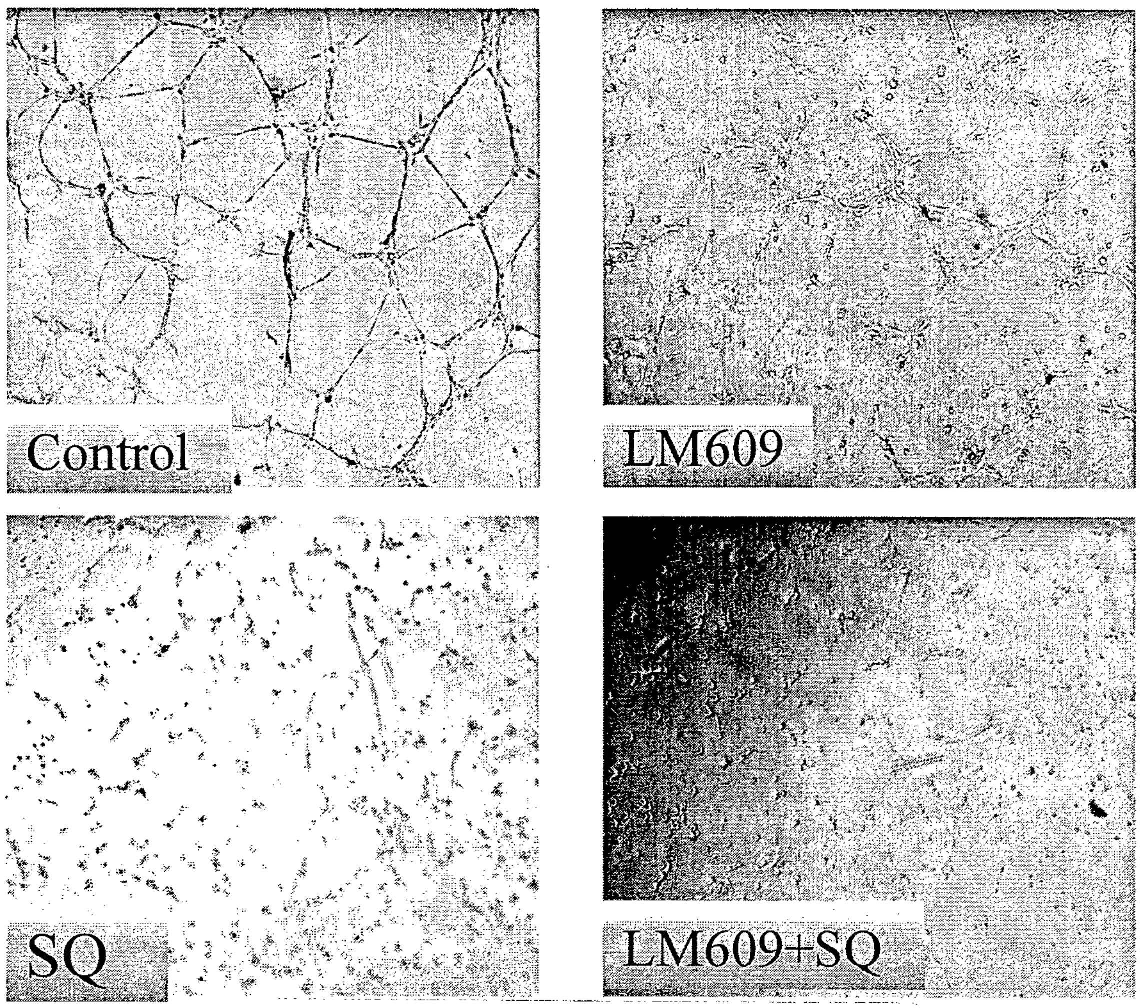

Figure 3

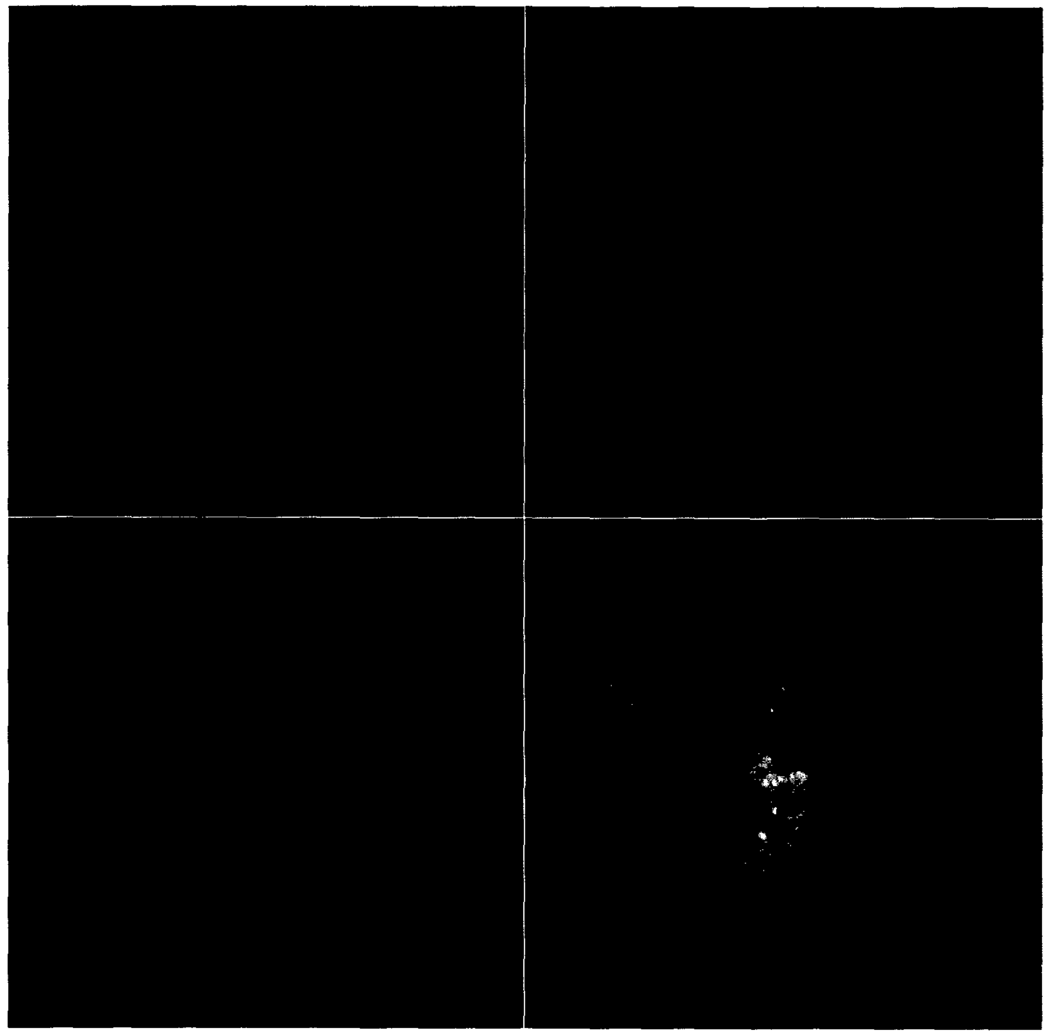
Prostate Stroma
LNCaP
HUVEC
LNCaP and HUVEC and Prostate Stroma

Figure 4

Development of Squalamine as an Anti-angiogenic Agent for the Treatment of Human Prostate Cancer in Experimental Models

Fengshuo Jin, Mitchell H. Sokoloff, Buer Sen, Jun Ling, Chia-Ling Hsieh, Haiyen E. Zhau and Leland W. K. Chung, Emory University School of Medicine, Atlanta, Georgia (FJ, BS, JL, C-LH, HEZ, LWKC) and University of Chicago, Chicago, Illinois (MHS)

Squalamine (SQ) is an anti-angiogenic aminosterol antibiotic isolated from shark liver. In previous studies, SQ has been demonstrated as a single effective agent in the treatment of solid tumors and interruption of embryonic vasculature. We showed that when applied in combination with *immediate* rather than *delayed* androgen withdrawal SQ increased cell-kill in mice bearing human prostate tumors [J. Urol. 1999, 161:298]. In this communication, we report upon a series of *in vitro* and *in vivo* investigations designed to determine the effects of SQ on the proliferation and migration of human prostate cancer cell lines and on the growth and tubular formation of a HUVEC endothelial cell line.

SQ was found to inhibit the growth of both human prostate cancer and HUVEC cells *in vitro* in a dose-related manner (HUVEC most sensitive, LNCaP/C4-2 intermediate and PC3, PC3M and DU145 least sensitive). Combined treatment of LNCaP/C4-2 cells (androgen receptor positive) but not PC3/DU145 (androgen receptor negative) with SQ and vascular endothelial growth factor (VEGF) markedly enhanced their cytotoxicity. AR withdrawal may play a role in upregulating VEGF receptor, Flt-1, expression which might contribute to the cytotoxicity induced by combined SQ and VEGF through downstream cell signaling.

SQ also exerted a concentration-dependent inhibition of HUVEC cell migration and tubular formation. When prostate tumor-bearing animals were treated *in vivo* with SQ immediately after castration, we observed that responding tumor tissues demonstrated over-expression of Flt-1 and integrin $\alpha6\beta4$ but decreased expression of integrin $\alpha v\beta3$ when compared to non-responding tumor tissue. This suggests a possible relationship between integrin expression and signaling and the sensitivity of prostate tumors toward SQ-induced cyotototoxicity. We have created a 3-D cell model that incorporates a color-coded mixture of tumor, endothelial and stromal fibroblast cells to study the effect of SQ and VEGF and have evaluated the role of focal adhesion kinase and the closely-related kinase Pyk2 in downstream signaling in this model. The overall effects of SQ in altering this signaling pathway will be discussed.

(Supported by DoD DAMD 17-00-1-0526 to LWKC and CapCURE Foundation to MHS)

Appendix 2

Differentiation (2002) 70:506–521

REVIEW

Shian-Ying Sung · Leland W. K. Chung

Prostate tumor-stroma interaction: molecular mechanisms and opportunities for therapeutic targeting

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Abstract Maintenance of cell and tissue homeostasis is dependent upon the dynamic balance of cell proliferation, differentiation, and apoptosis through interactions between cells and their microenvironment. The unique prostatic cellular phenotypes are induced and maintained by interaction between epithelium and adjacent stroma through intimate intercellular signaling pathways. In this article, we summarize current advances in the tumor-stroma interaction and its biologic and therapeutic implications. We specifically emphasize current studies of the possible factors driving the "vicious cycle" between stroma and emerging prostate tumor epithelial cells that may be responsible for carcinogenesis and metastasis to bone. Stroma responds both genotypically and phenotypically to tumor epithelium upon co-culture under 3-D conditions. Likewise, the emerging carcinoma responds to stromal signals that drive progression to malignancy. A vicious cycle mediated by soluble and insoluble molecules secreted by tumor cells and stroma appear be the critical factors supporting and sustaining tumor colonization in bone. Co-targeting tumor and stroma with therapeutic agents has yielded promising results both in pre-clinical models of prostate cancer and bony metastasis and in clinical trials of patients treated with a dual tumor and stroma targeting strategies. In conclusion, understanding and targeting the interaction of the tumor and its stromal microenvironmant may improve the prognosis, reduce the suffering and increase the survival of patients with advanced cancer metastasis.

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Key words prostate cancer · bone metastasis · stromal-epithelial interaction · molecular targeting · vicious cycle · cytokine · extracellular matrix (ECM)

Introduction

This review will first elucidate the molecular mechanisms underlying prostate tumor-stroma interaction, involving prostatic stromal cells at the primary site and osteoblasts and osteoclasts at bone metastasis sites, and then discuss new opportunities for therapeutic targeting of localized and disseminated human prostate cancer.

Cell and tissue homeostasis reflects a dynamic balance of cell proliferation, differentiation and apoptosis (Frisch and Screaton, 2001). Consistent with this concept, primary and immortalized non-transformed human prostate epithelial cells require adhesion to an extracellular matrix (ECM) to maintain their polarity, growth, survival, and migratory characteristics and expression of tissue-specific proteins. These properties are unique organ-specific phenotypes conferred and maintained by interaction between epithelium and adjacent ECM secreted primarily by the stroma through intimate intercellular signaling pathways. Epithelial cells, the major target for adult cancer, exist in contiguous sheets composed of organized, polarized cells circumscribed by a basement membrane that separates the epithelium from the stroma. Numerous cell types are found in the epithelial and stromal compartments, including luminal, basal, and neuroendocrine cells in the epithelial tissue compartment and smooth muscle, fibroblast, endothelial, neuroendocrine, neural, and inflammatory cells in the stromal tissue compartment. Intercellular interaction between these cell types, mediated by soluble factors and insoluble ECM, will determine the growth and differentiation potentials of the entire organ.

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Homeostasis of normal organs such as prostate and breast is maintained through reciprocal interactions between epithelial cells and their surrounding stroma with minimal proliferation of either cell type. Disruption of the homeostatic interaction between epithelium and stroma could initiate and promote carcinogenesis. In these instances, carcinogenic insults may trigger additional genetic changes in the epithelial cell compartment over and beyond the inherited traits, through increased genomic instability and decreased DNA repair and apoptotic signaling. Altered epithelial cells may trigger stromal reactions that, in turn, confer reciprocal signal cascades in tumor epithelium to promote further carcinogenic processes. Ultimately, reciprocal tumor-stroma interaction culminates in the increased migratory, invasive, and metastatic behavior of cancer cells.

Prostate carcinoma-stroma interaction

Prostate epithelium and stroma are sites for the development of benign and malignant diseases of the prostate. Recent evidence suggests that prostate epithelium and stroma interact in a highly organ-specific, androgen-dependent and temporally-related manner. We discuss below the role of prostate fibromuscular cells in prostate tumor growth and progression, the reciprocal stromal reactions to prostate tumor epithelium that create a "vicious cycle" between stroma and epithelium ultimately driving tumor epithelium to develop benign and malignant prostate diseases, the potential factors that may mediate these interactions, and the role of early prostate inflammatory atrophy in the development of benign prostatic hyperplasia and prostate cancer.

Role of prostate fibromuscular stromal cells in prostate tumor growth and progression

In 1970, Professor L. M. Franks described the requirement of prostate cancer fibromuscular stromal cells for the growth and survival of primary human prostate epithelial cells in culture (Franks et al., 1970). We tested the significance of this original *in vitro* observation in our laboratory by both *in vitro* cell co-culture and *in vivo* co-inoculation of tumor cells and stromal cells in immune-compromised mouse models for the growth of human and rat prostatic tumors as xenografts. A series of reports demonstrated that the growth of benign and cancerous prostate epithelial cells in vivo was enhanced markedly by the co-presence of organ-specific and/or cancer-associated stromal cells (Chung et al., 1989; Camps et al., 1990; Gleave et al., 1991; 1992). These studies were confirmed by other laboratories which showed that prostate tumor growth *in vivo* could be accelerated by cancer, but not by benign tissue-associated stromal fibroblasts (Olumi et al., 1998; 1999; Wong and Wang, 2000).

Further, the androgen-independent and metastatic progression of human prostate epithelial cells can be promoted by co-inoculating a marginally tumorigenic human prostate cell line, LNCaP, with a human bone stromal cell line derived from an osteosarcoma *in vivo* (Thalmann et al., 1994; 2000; Wu et al., 1994; 1998). By a series of manipulations of chimeric LNCaP tumor growth *in vivo* under the influence of bone stromal cells, either in the presence or absence of androgen, the derivative LNCaP sublines C4–2 and C4–2B acquired the ability to become androgen-independent and metastatic, as exhibited by their behaviors in immune compromised mice. To ascertain that cell-cell contact rather than unknown factors from the host were responsible for conferring tumorigenic and metastatic potential to the parental LNCaP cells, we co-cultured LNCaP cells with either prostate or bone stromal cells under 3-dimensional (3-D) conditions and observed similar permanent phenotypic, genotypic and behavioral changes of the parental LNCaP cells, as revealed by their ability to form tumors in castrated mice and acquired ability to metastasize to distant organs including bone (Ozen et al., 1997; Pathak et al., 1997; Rhee et al., 2001).

These results taken together suggest that tumor stroma can confer "inductive" or "adaptive" cues to the responding tumor epithelium and is directly responsible for the altered behavior of tumor epithelium. However, tumor-stroma interaction is reciprocal. Not only can stroma "induce" or "select" the phenotypic and genotypic changes in tumor epithelial cells, tumor epithelium can also induce genetic and phenotypic changes in stroma after tight association *in vivo*. It appears that ***both*** tumor and stroma are involved in controlling tumor growth or " take" by the host and the subsequent progression of tumor epithelium to androgen-independence and acquisition of local invasive and distant metastatic potential in experimental models of human prostate cancer. Figure 1 depicts a model of the multi-step nature of this interaction. Genetically and phenotypically altered epithelial cells induce a stromal reaction that, in turn, induces a reciprocal epithelial reaction. The serial interactions form a "vicious cycle" that drives epithelial cancer progressive to androgen-independent local invasion and distant metastasis.

Through growth factor activation, changes in ECM can be elicited, which can cause epithelial cells to lose their apical-basal polarity and thus assume a less well differentiated state (Bissell and Radisky, 2001). This dramatic alteration of epithelial cell phenotype can lead to increased cell proliferation and tumorigenesis (Reichmann, 1994; Naishiro et al., 2001).

However, the specific molecules responsible for tumor-induced changes in the microenvironment and the reciprocal modifications of the tumor by its microenvironment are largely unknown, as are the inter- and intra-cellular pathways that result from these influences. Dissecting the components of the stroma requires model

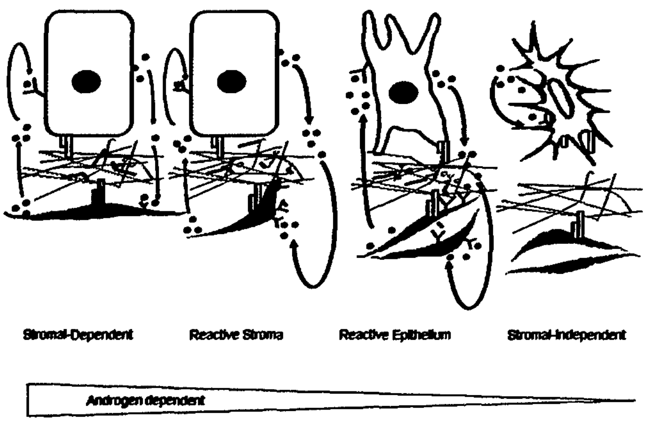

Fig. 1 "Vicious cycle" of prostate cancer bone metastasis. This figure describes the growth factor and extracellular matrix-mediated activation of transcription factors, which control the matrix proteins, and MMPs activation. The vicious cycle may be initiated first by the presence of growth factor/ECM milieu in the prostate cancer which up-regulates key transcription factors that modulate matrix and metalloproteinase expression in stromal cells. Increased expression of chemokines, cytokines, and transcription factors can activate of additional growth factors and ECM pathways, which drive prostate tumor cells to a more invasive and malignant state.

systems in which a single variable can be manipulated and assessed. In contrast to tumor-associated stroma, normal stromal cells have a low proliferative index, probably secrete only the factors necessary to maintain normal tissue function (Manabe and Owens, 2001), and appear to be less responsive to inductive cues elaborated from normal epithelium.

Stromal reaction to tumor epithelium

Since stromal cells from normal adult tissues are less inductive, or often non-inductive, the experimental data imply that stromal cells exposed to tumor epithelium could be "activated" and acquire an inductive potential to drive the subsequent neoplastic processes. This suggestion is supported by several lines of evidence.

First, morphologic "desmoplastic" stromal response to tumor epithelium often occurs around either primary or metastatic tumor epithelium (Thompson et al., 1993; Nemeth et al., 1999; Tuxhorn et al., 2001; 2002). A desmoplastic stromal response is characterized by increased proliferation of fibromuscular stromal cells and enhanced deposition of extracellular matrices (ECMs) surrounding tumor epithelium. This active process could be viewed as a part of the host-defense mechanism to curtail or restrict tumor expansion. Conversely, this reaction and accompanying increased stromal cell number could provide a "fertile soil" supporting the growth and invasion of tumor epithelium through the increased production by stromal cells of growth factors and stroma-associated ECMs. In addition, stromal cells are the major production sites of metalloproteinases, which increase ECM turnover and are thought to be critical to the invasive property of tumor epithelium. Thus, global changes in the tumor microenvironment could provide selective growth and survival advantages for certain tumor cell clones, particularly in androgen-deprived conditions.

Second, stromal reaction to tumor epithelium may be *irreversible*, if the reacting stromal cells receive an "inductive cue" from tumor epithelium to undergo trans-differentiation, whereby stromal fibroblasts adjacent to tumor epithelium convert both morphologically and phenotypically to myofibroblasts (Ronnov-Jessen et al., 1995; Elenbaas and Weinberg, 2001; Tuxhorn et al., 2001, 2002).

Transition of stromal fibroblasts to myofibroblasts with increased expression of vimentin pro-collagen type-I and tenascin has been observed (Tuxhorn et al., 2001). Using the laser capture microdissection (LCM) technique, genetic aberrations were detected in the fibromuscular stromal compartment surrounding tumor epithelium, further supporting the reciprocal nature of the tumor-stroma interaction (Moinfar et al., 2000). Although the mechanisms of the genetic and phenotypic response of stroma to adjacent tumor epithelium are currently unclear, possible mechanisms include: a) Transition or inter-conversion of epithelium to stroma (Wernert et al., 2001); b) Irreversible induction of stromal changes, both at the morphologic and the biochemical levels, by soluble and insoluble factors secreted by tumor epithelium; c) Selection of previous existing clones of stromal cell populations and preferential expansion of these clones based on their proliferative and survival advantages (Singer et al., 1995; Tso et al., 2000); d) The combination of b and c above; that is, after prolonged "adaptation" to a tumor-associated stromal microenvironment, permanent genetic changes may occur in the stromal cell population through a poorly understood "adaptive mutation" mechanism (Laval and Laval, 1984; Chung, 1995).

A "vicious cycle" between prostate stroma and tumor may be responsible for carcinogenesis in primary prostate cancer

Studies using tissue and cell recombination models demonstrate that growth and differentiation of the prostate gland depends on reciprocal cellular interaction between prostate epithelium and its adjacent stroma (Chung et al., 1991; Cunha et al., 1996; Wong et al., 1998; Wong and Wang, 2000). Evidence also suggests that androgen receptor in the stroma rather than in the epithelium may be critical for conferring the growth and differentiation functions of the prostate gland (Cunha and Chung, 1981; Thompson and Chung, 1984). When normal prostate epithelium (Zhau et al., 1994; Chung, 1995) or urothelium (Zhau et al., 1994) was used in these studies, the

inductive fetal urogenital mesenchyme determined the ultimate size of the tissue-tissue recombinant. However, when prostate tumor tissues (Chung et al., 1984; Miller et al., 1985) or tumor cells derived from the prostate (Gleave et al., 1991; 1992) or urinary bladder (Zhau et al., 1994) were used in the experiments, the growth of the tissue-tissue or tissue-cell recombinants was uncontrolled and never reached a state of homeostasis.

One interpretation of these results is that signaling between tumor and stroma is aberrant and resembles a "vicious cycle" where a dysfunction of cytokine trafficking exists between tumor and host cells (Mundy et al., 2001; Tester et al., 2002). There are several possible mechanisms for the activation of a vicious cycle between tumor and stroma: 1) tumor cells secrete putative cytokines, growth factors and/or extracellular matrices that alter the morphology and gene expression of surrounding stroma such that the altered stroma becomes highly inductive and reciprocally induces the growth and gene expression of tumor epithelium, thus initiating the vicious cycle. Rowley and collaborators provided evidence that stromal fibroblasts surrounding tumor epithelium underwent trans-differentiation to become a morphologically and biochemically distinct population of myofibroblasts (Rowley, 1998; Tuxhorn et al., 2001; 2002). Interestingly, they have shown that this type of stromal response to tumor epithelium can predict PSA-free survival in patients with prostate cancer. 2) Tumor cells secrete soluble factors that act in an autocrine manner to promote the vicious cycle regardless of the surrounding stroma. Under certain stress and androgen conditions, increased growth factors, such as vascular endothelial growth factor (VEGF) production by tumor cells, have been observed (Wong et al., 1998; Jackson et al., 2002). Increased VEGF was shown to induce more oxygen stress and initiate the vicious cycle by promoting more VEGF production by tumor cells, eventually causing an accumulation of neovasculature surrounding the tumor epithelium (Ferrer et al., 1997; 1998; Burchardt et al., 2000; Arbiser et al., 2002; Colavitti et al., 2002). 3) The intrinsic genetic instability of tumor cells can be promoted by tumor-microenvironment interaction (Rhee et al., 2001; Tlsty, 2001). It is conceivable that increased cytogenetic changes, with loss or gain of growth control genes, could fuel additional genetic instability not only of tumor cells *per se* but also of their surrounding stroma (Moinfar et al., 2000).

Potential factors responsible for activating prostate carcinogenesis and driving the "vicious cycle" of prostate stroma and tumor

Integrins

Integrins are important in prostate cancer progression and metastasis. The major role of integrins in cancer is the "outside-in" pathway, in which integrin activation induces cancer cell migration and invasion. Integrins also cooperate with growth factors to promote cell proliferation. When adherent tissue cells are released from their surrounding extracellular matrix, they forfeit survival signals and undergo apoptosis (Porter and Hogg, 1998). In addition to interacting with stromal cells or ECM, integrins can also form cis associations with other receptors on the same cell, forming multi-receptor complexes. These complexes recruit signaling molecules to sites of cell-cell or cell-matrix adhesion, such as focal complexes and focal adhesions (Edlund et al., 2001). Integrins also play a crucial role in regulating the actin cytoskeleton at the site of contact with ECMs. Although the detailed pathway is still not very clear, data show that once integrins receive signals from ECMs they can turn on other genes such as α-actin, talin, vinculin and vasodilator-stimulated phosphoprotein. Signaling through Rho family pathways could activate Cdc42, Rac and Rho genes which further turn on downstream signaling pathways such as the calpain and JNK pathways. Integrins also could regulate the activation of the focal adhesion kinase pathway, Src protein tyrosine kinase, and paxillin, which are important in the remodeling and turnover of adhesion complexes (Martin et al., 2002).

Modulation of integrin activation is closely linked to gene expression, cell cycle progression and cellular behaviors, such as cell motility, migration, and survival under various physiologic and pathologic conditions. The increased expression of $\alpha 3$ and $\beta 6$ integrins compared with normal cells has been demonstrated. The expression of $\alpha 6\beta 1$ integrin on prostate cancer cells was linked to increased invasion of prostate cancer in a mouse model (Schmelz et al., 2002). We and other groups have studied cell interactions with extracellular matrix and stromal factors during disease progression by characterizing integrin usage and expression in a series of parental and lineage-derived LNCaP human prostate cancer cell lines (Cress et al., 1995; Allen et al., 1998; Bello-DeOcampo et al., 2001; Edlund et al., 2001; Cooper et al., 2002; Schmelz et al., 2002). Although studies indicated the decrease of integrin heterodimers, the actual integrin expression on the cell surface showed no significant change; however, with disease progression, integrin usage did change significantly. The more metastatic sublines were distinct in their use of $\alpha v\beta 3$ integrin (Edlund et al., 2001). When compared with parental LNCaP cells, the more metastatic sublines showed a shift in $\alpha 6$ heterodimerization, a subunit critical not only for interaction with prostate basal lamina but also for interaction with bone matrix proteins, a favored site of prostate cancer metastases (Edlund et al., 2001). This indicates that integrin usage changed during the progression of prostate cancer. The activation state of integrins could be an important element in how cells adapt under different microenvironmental conditions. The adaptive property of integrins could be enhanced further

with changes in mediators or ECMs during the metastatic progression of prostate tumor epithelial cells, a potential step toward migratory properties.

The αvβ3 integrin heterodimer has been detected on many different cell types, such as macrophage, endothelial cells, osteoclasts, and cancer epithelial cells. The activation of αvβ3 in prostate cancer cells is mediated by the FAK pathway that activates the downstream PI-3 kinase/Akt pathway (Zheng et al., 2000). This triggers alterations in cell adhesion and migration on a variety of extracellular matrix proteins, including vitronectin, fibronectin, fibrinogen, laminin, collagen, and osteopontin. αvβ3 has been shown to be important for prostate cancer bony metastasis by adhesion of cancer cells to bone matrix components, vitronectin, osteopontin, and bone sialoprotein (BSP). Through analysis of DU145 cell adhesion to ECM, Zheng and colleagues showed that the adhesive property of DU145 cells can be decreased by LM609, a blocking avb3 antibody (Zheng et al., 1999; 2000). Osteopontin and vitronectin are common proteins in mature bone and can potentially serve as ligands for αvβ3.

Integrin-associated protein (IAP/CD47) is a 50 kDa single-chain protein composed of an extracellular immunoglobulin superfamily (IgSF) domain, five membrane-spanning sequences and a short cytoplasmic tail. IAP was first isolated as a protein associated with the integrins, αvβ3, αIIbβ3, αvβ5, and α2β1. Human cells that lack IAP are deficient in avb3mediated ligand binding (Porter and Hogg, 1998). In an affinity purification study in which the 179–208 peptide of the α3(IV) chain of collagen IV was used as the immobilized element, five proteins from melanoma and prostate cells were isolated. The 3 proteins were shown to be CD47/IAP, the integrin b3 subunit, and the αvβ3 integrin complex, respectively (Shahan et al., 1999), indicating that avβ3 and IAP formed a complex in prostate cancer cells that could activate the functional property of αvβ3 in prostate cancer epithelial cells. Another recent study indicated that avβ3 inhibits endothelial cell apoptosis during angiogenesis through NF-κB activation (Cooper et al., 2002). Angiogenesis facilitates the growth and metastasis of tumors by providing support and facilitating cancer migration. Together these studies suggest that αvβ3 is important not only for the growth and survival of tumor epithelium but for its supporting endothelium.

Growth factors

The expression of *basic fibroblast growth factor (bFGF, FGF-2)* has been shown to be significantly increased in stromal fibroblasts in human prostate cancer and in endothelial cells compared with normal tissue. Prostate carcinoma cells have been shown to up-regulate fibroblast growth factor receptor isoforms with a high affinity for bFGF during cancer progression (Dow and deVere White, 2000). Accordingly, elevated sensitivity to bFGF may stimulate cancer cell proliferation and protease expression, thereby supporting tumor growth and invasion. In addition, overexpression of both FGFR-1 and FGFR-2 in prostate cancer epithelial cells in a subset of prostate cancers has been correlated with poor differentiation. Thus, there is both an increase in bFGF concentration in stromal cells and increased expression of receptors in tumor epithelial cells which respond to bFGF, establishing a potential paracrine loop between prostate cancer cells and their surrounding stromal cells, which may be important for prostate cancer progression (Giri et al., 1999).

bFGF also stimulates fibroblast proliferation and extracellular matrix turnover through increased deposition and protease degradation (De Benedetti and Harris, 1999; Dow and deVere White, 2000), and functions as an angiogenic factor that induces endothelial cell migration, proliferation, and differentiation into new blood vessels (De Benedetti and Harris, 1999). Thus, bFGF may promote prostate cancer progression by inducing angiogenesis and stromal remodeling. A study also indicates the increase of bFGF in tumor epithelial cells due to induction of stromal FGF-2 (Giri et al., 1999), thus potentially establishing a positive feedback loop. Human prostate cancer cell lines DU-145 and PC-3 have been shown to express FGF-2 and metastasize to bone (Dow and deVere White, 2000). Furthermore, studies of the Dunning rat model show that activation of bFGF expression accompanied progression of epithelial cells to malignancy (Yan et al., 1993). These data suggest a possible contributing role for bFGF in the vicious cycle of tumor formation and progression.

Platelet-derived growth factor (PDGF) is a 30-kD protein consisting of disulfide-bonded homodimers or heterodimers of A and B subunits, also designated as c-sis (Sitaras et al., 1988). Its isoforms have been indicated as important during embryonic development, particularly in the formation of connective tissue in various organs (George, 2001). In adult tissues, the primary function of PDGF is to stimulate wound healing via chemotaxis and mitogenesis of fibroblasts, and secretion of extracellular matrix components (Tuxhorn et al., 2001). The normal physiologic targets for PDGF are stromal cells such as fibroblasts, endothelial cells, smooth muscle cells, and glial cells. Thus, paracrine release of PDGF stimulates stromal reactions in normal and pathologic states. Receptor binding by PDGF is known to activate intracellular tyrosine kinase, leading to autophosphorylation of the cytoplasmic domain of the receptor as well as phosphorylation of other intracellular substrates. This reaction is described as one in trans, i.e., the two receptor molecules of the receptor dimer phosphorylate each other. Specific substrates identified with the beta-receptor include Src, GTPase Activating Protein (GAP), phospholypase C (PLC) and phosphotidylinositol 3-phosphate. Both PLC-γ and GAP seem to bind with different affin-

ities to the α- and β-receptors, suggesting that the particular response of a cell depends on the type of receptor it expresses and the type of PDGF dimer to which it is exposed. In addition to the above, a non-tyrosine phosphorylation-associated signal transduction pathway can also be activated that involves the zinc finger protein Erg-1 (Khachigian and Collins, 1998).

Immunohistochemical analysis of PDGF and PDGFR indicated expression in both prostate epithelial and stromal cell types and in PIN lesions (Fudge et al., 1996). In contrast, the normal epithelial cells do not express PDGF nor PDGFR (Fudge et al., 1994). *In vitro* study of PDGF indicates that release of PDGF from tumor cell lines stimulates prostate stromal cell proliferation (Vlahos et al., 1993). This suggests that *de novo* expression of PDGF occurs early in prostate tumor progression. The production and activation of PDGF could further enhance the stromal reaction and contribute to the vicious cycle of tumor progression.

Recruitment of new blood vessel growth clearly illustrates the importance of tumor-stromal interactions during cancer progression. In normal human prostate tissue, *VEGF* is reportedly expressed at low levels and restricted to stromal cells. In high-grade PIN and prostate cancer, elevated expression of VEGF was observed in cancer, stroma and vascular endothelium (Ferrer et al., 1997). Endothelial cells from microvessels in the surrounding stroma must be induced to migrate into the tumor, whereby they proliferate and form new blood vessels to support tumor growth. This complex process is regulated by a delicate balance of angiogenesis inducers and angiogenesis inhibitors in the extracellular milieu. Increased activator(s) and/or decreased inhibitor(s) alter the balance and lead to the growth of new blood vessels (Hanahan, 1997). Several growth factors, such as VEGF, PDGF, TGF-β and connective tissue growth factor, from epithelium or stroma, could induce angiogenesis (Nadal et al., 2002).

Recent studies indicate that VEGF directly stimulates prostate tumor cells via autocrine and/or paracrine mechanisms (Sokoloff and Chung, 1998; Jackson et al., 2002). One example demonstrated the possible role of VEGF as a mediator in the vicious cycle of tumor and stroma, in which reactive oxygen species (ROS) could participate in early prostate cancer epithelium growth and development. Increased ROS could enhance the production of VEGF, further promoting ROS concentration in stromal fibroblasts. The resulting overexpression of VEGF from stromal fibroblasts could induce Nox1, MMP-9, VEGF, and VEGFR production and increase the overall tumor growth rate (Arbiser et al., 2002).

In normal prostate tissue, *IGF-1* is produced only by stromal cells, while prostate epithelial cells express insulin-like growth factor binding proteins (IGFBP-2, 3, 4, and 6) and the type 1 IGF receptor (Lopaczynski et al., 2001). It has been shown that both bFGF and PDGF can enhance IGF-1 production from endothelial cells. Prostate cancer patients have shown increased serum IGF-1 and a decrease of IGFBP-3 level (Mantzoros et al., 1997; Chan et al., 1998; 2002; Chokkalingam et al., 2002; Grimberg et al., 2002). The increased serum IGF-1 concentration in prostate cancer patients could be from the stromal cells, metastatic prostate cancer epithelial cells or the liver. However, the study of IGF secretion is controversial. Some reports did not detect IGF-1 production by prostate cancer cells (Cohen et al., 1991; Pietrzkowski et al., 1993; Connolly and Rose, 1994; Angelloz-Nicoud and Binoux, 1995), others indicated the expression of IGF by prostate cancer cells (Iwamura et al., 1993; Kimura et al., 1996; Kaplan et al., 1999). Interactions between the glandular epithelium and the myofibroblasts and fibroblasts of the stromal compartment of the prostate gland appear to be regulated by IGF-1 *availability.* IGF-1 may act directly through the androgen receptor pathway and may be regulated through EGF-TGF-α receptor regulatory signaling (Kimura et al., 1996). This suggests the possible vicious cycle of IGF-1 production in prostate cancer progression. First, release of bFGF and PDGF induces IGF-1 secretion from prostate stromal cells, which could induce the increased production of androgen receptor and/or EGF from prostate tumor epithelial cells. The enhanced expression of the androgen receptor and elevated release of EGF can, in turn, further stimulate the release of IGF-1 from stromal cells, which may promote the progression of prostatic carcinoma cells.

Two huge molecules called *plasminogen-related growth factors (PRGFs)*, evolutionarily related to plasminogen, play an important role in inducing invasive growth of cancer progression. PRGF-1 is also called *hepatocyte growth factor/scatter factor (HGF/SF)*. PRGF-2 is also known as *macrophage-stimulating protein (MSP)*, *scatter factor-2* (Comoglio et al., 1999). HGF/SF has been demonstrated to be important in prostate cancer progression and metastasis, while MSP may be an important neurotrophic factor in embryonic development by inducing superoxide anion production (Brunelleschi et al., 2001; Rampino et al., 2002). It has been shown that both HGF/SF and MSP were up-regulated in the wound repair process in a rat model (Cowin et al., 2001). HGF/SF predominantly participates in a paracrine network. Several mesenchymal-derived cells (fibroblasts) secrete HGF/SF. It has been implicated as a mediator involved in communication between epithelial cells and the microenvironment (Comoglio and Trusolino, 2002). HGF/SF is secreted predominantly by stromal fibroblasts and stimulates proliferation and migration of epithelial and endothelial cells during organ development and tissue remodeling (Parr and Jiang, 2001). The secretion of HGF/SF as an inactive pro-HGF, which is converted into its bioactive form by a proteolytic cleavage by four proteases: urokinase (uPA), serine protease in the serum, coagulation factor XII, and its homologues (Comoglio et al., 1999). It has also been shown that some epithelial

cells secrete two potent inhibitors of pro-HGF activation (HAI-1 and -2) that tightly control HGF/SF activation (Denda et al., 2002).

In normal prostate epithelial cells, HGF/SF secreted by stromal cells causes growth inhibition, sustained phosphorylation of mitogen-activated protein kinase, and increased gene expression consistent with cell differentiation. Several soluble factors increase the production of HGF/SF in myofibroblasts but not in normal prostate epithelial cells, such as IL-1β, PDGF, bFGF, VEGF, and EGF (Zhu and Humphrey, 2000). Increased expression of the HGF/SF receptor c-Met proto-oncogene has been associated with progression of several types of carcinoma, including that of the prostate (Humphrey et al., 1995; Pisters et al., 1995; van Leenders et al., 2002). As mentioned above, the vicious cycle of bFGF, PDGF, and VEGF could further mediate or enhance the secretion of HGF/SF from stromal cells. Several studies have indicated that the increased concentration of HGF/SF in ECM could further contribute to malignancy in DU145 or PC-3 prostate tumor cells, inducing migration (Gmyrek et al., 2001; Nishimura et al., 1999).

Cytokines

In a normal homeostatic state, *IL-6* levels are typically very low. However, in response to microenvironment inflammatory factors, IL-6 can be released by wide variety of cell types. Cells known to express IL-6 include CD8+ T cells, fibroblasts, synoviocytes, adipocytes, osteoblasts, megakaryocytes, endothelial cells (under the influence of endothelins), neurons, neutrophils, monocytes, colonic epithelial cells, and B cells. IL-6 production is generally correlated with cell activation. Studies of tumor stroma indicate that increased IL-6 could induce the progression of prostate tumor epithelial cells by inducing the release of other cytokines, bone resorption, and induction of thrombopoiesis (Smith et al., 2001). Several groups have reported elevated serum levels of IL-6 upon progression of prostate cancer to androgen-independence (Nakashima et al., 2000; Shariat et al., 2001). It is possible that mediators released from prostate tumor epithelial cells could further enhance the production of IL-6 from both stromal and inflammatory cells. The increase concentration of IL-6 in ECM may further induce prostate tumor epithelial cells to produce mediators and IL-6R. Once prostate cancers reach malignancy, the tumor epithelial cells can produce IL-6 themselves and form an active autocrine loop.

A recent study indicated the increase of both IL-6 and IL-6R in prostate tumor epithelial cells with the increase of malignancy (Giri et al., 2001), again suggesting a "vicious cycle" mediated by IL-6 during the early development of prostate cancer and becoming an increasingly active autocrine loop in highly metastatic tumors. Similar vicious cycles have been shown for other cytokines, such *IL-8*. In human prostate cancer, IL-8 has been shown to stimulate PC-3 prostate cancer cell migration and invasion *in vitro* through a reconstituted basement membrane and both long-term migration and short-term adhesion to laminin (Reiland et al., 1999). IL-8 is produced by many different stromal cells, including endothelial cells. IL-8 is also produced by various metastatic tumor cells, including prostate cancer cells (Kim et al., 2001). Furthermore, stress factors, such as hypoxia, acidosis, nitric oxide (NO), and cell density, which increase with the progression to malignancy, can also influence IL-8 production (Shi et al., 2000).

Stromal response to early prostate inflammatory atrophy

Inflammatory reactions often result in the activation and recruitment of phagocytic cells (e.g., neutrophils and/or tissue macrophages) whose products, such as cytokines, oxidants and free radicals, result in injury to the tissue. Recent reports indicate that benign prostatic hyperplasia (BPH) frequently exhibits infiltration of CD4(+)/CD45RO(+) memory T-lymphocytes. This infiltration could induce growth of myofibroblast cells in BPH. Increased level of cytokines, such as IL-2, IL-4 and TNF, were also detected in T-cells in BPH but not in normal prostate (Kramer et al., 2002). Another study also indicated the association of inflammation with BPH and prostate cancer, and the increased expression of Bcl-2 in these prostate patients (Gerstenbluth et al., 2002). The chronic inflammation status linked to the development of tumor has been reported in several organ systems, including prostate cancer (De Marzo et al., 1999). The hypothetical mechanism involves repeated tissue damage and regeneration in the presence of highly reactive oxygen. These reactive molecules, i.e. hydrogen peroxide (H_2O_2) released from the inflammatory cells interact with DNA in the proliferating epithelium to produce permanent genomic alteration, such as frame-shift mutation, deletions, and rearrangements, as well as increasing the epithelial proliferative rate (Gasche et al., 2001; Oda et al., 2001). Recent studies of prostate cancer indicated inflammatory responsive cells at the juxtaposition of highly proliferative prostate epithelial cells, referred to as a Proliferative Inflammatory Atrophy lesion (PIA lesion). Studies demonstrated mononuclear and/or polymorphonuclear inflammatory cells in both the epithelial and stromal compartments, and stromal atrophy with variable amounts of fibrosis. Luminal epithelial cells of PIA lesions have elevated levels of Bcl-2, decreased expression of $p27^{Kip1}$, and increased levels of π-class glutathione S-transferase (GSTP1) (De Marzo et al., 1999). *In vivo* study of H_2O_2 and GSTs indicated that H_2O_2 enhances the expression of GSTP1 (Liu et al., 2001). This indicates that increase expression of GSTs in PIA may be due to increased concentrations of H_2O_2 in the stromal microenvironment during

the PIA stage. However, PIN and prostate cancer cells rarely express GSTα isoenzyme (GSTA1) and GSTP1 as a result of increased methylation of GSTP1 in a "CpG island" which inactivates GSTP1 (De Marzo et al., 1999; Parsons et al., 2001).

The phenotypic switch of stromal cells, extracellular matrix remodeling, increased growth factor availability, elevated protease activity, increased angiogenesis, and recruitment of inflammatory cells were observed in cancer progression. The stromal response to cancer shows similarity to the wound repair response (Tuxhorn et al., 2001), and it is possible that these conditions could promote tumorigenesis. The phenotypic switch between fibroblast and myofibroblast indicates increased extracellular matrix remodeling during prostate cancer progression. In normal prostate it has been reported that the "stromal network of collagen fibers is loosely woven, fine and smooth in texture," while in Gleason-score seven adenocarcinoma the collagen fibers "appeared swollen in diameter" and there was "no regularity in the spatial relationship of the fibers" (Keller et al., 2001; Tuxhorn et al., 2001). This suggests that remodeling of the extracellular matrix is one of the key features of stromal reaction in prostate cancer.

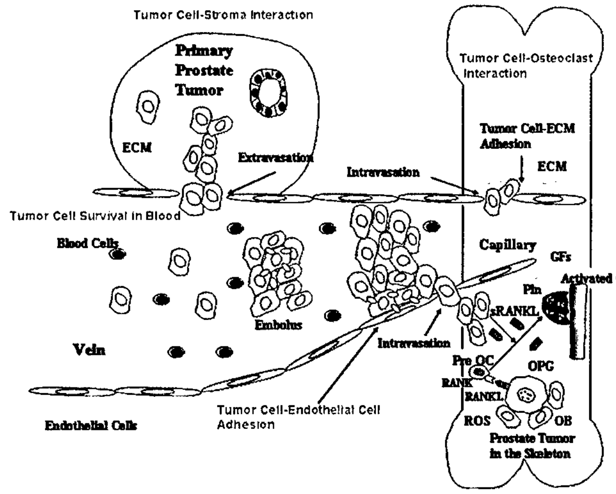

Fig. 2 The multi-step processes of prostate cancer metastasis to bone. Once prostate cancer cells gain the ability to extravasate into the bloodstream, prostate cancer cells move as embolus prior to adhering to bone marrow-associated endothelial cells. The attachment and interaction of prostate cancer cells to marrow endothelial ECMs could activate the invasive properties of prostate cancer cells and allow their extravasation into the marrow space. Prostate cancer cells than interact directly with osteoblast and osteoclast through a series of soluble factors via cell surface receptor (e.g., RNAK) to invade and replace the bone marrow components.

Prostate cancer and bone stromal interaction

The progression of prostate cancer from the androgen-dependent to the androgen-independent and bone metastatic state is considered a poor and generally lethal prognosis.. To understand the molecular basis of disease progression and develop rational new therapeutic approaches for targeting prostate cancer bone metastasis, we must first understand the multi-step processes that lead to prostate cancer metastasis to bone. As depicted in Fig. 2, at the site of primary cell growth we expect prostate cancer cells to interact with prostate stromal cells and gain the ability to extravasate into the bloodstream. In the blood, prostate cancer cells are expected to survive and move as an embolus prior to adhering to bone marrow-associated endothelial cells. The attachment and interaction of prostate cancer cells to marrow endothelial ECMs could activate the invasive properties of prostate cancer cells and allow their extravasation into the marrow space. At the final step of this progression, prostate cancer cells interact directly with osteoblasts and osteoclasts through a series of soluble factors (e.g., receptor activator of NF-κB ligand, RNAKL) via cell surface receptor (e.g., RNAK) to survive, proliferate, migrate and invade and eventually replace the bone marrow components. To understand the cellular and molecular basis of the prostate tumor-bone stroma interaction, it is essential to delineate how the soluble growth factors and extracellular matrices participate reciprocally in the progression of prostate cancer toward androgen-independence and bone metastasis.

A conceptual framework will be introduced here to illustrate the following issues. 1) Bone stromal reaction to cancer epithelium may signal further tumor progression. Altered bone stromal cells, in response to tumor epithelium, may induce further epithelial genetic and phenotypic changes and thus contribute to a vicious-cycle cascade in the androgen-independent and metastatic progression of prostate cancer (Fig. 1). 2) Co-targeting tumor and stroma could starve or kill tumor cells from their supporting microenvironment and could offer the greatest benefits for inducing tumor regression and sustaining the long-term survival of patients with prostate cancer skeletal metastasis and its associated complications.

By using a human prostate cancer co-culture model, our laboratory has obtained evidence suggesting that non-random genetic changes occur in human bone stromal cell line MG-63, after co-culturing with the human androgen-independent prostate cancer cell line C4–2 (a lineage-derived LNCaP subline with growth and metastatic potential to lymph node and bone when injected subcutaneously or orthotopically in castrated mice) under 3-D conditions. The 3-D model is valuable for the evaluation of the prostatic tumor-bone stroma interaction in vitro. The participation of bone stroma in tumor growth and progression suggests that when prostate cancer metastasizes to bone, there are complex and reciprocal cellular interactions between populations of tumor and host bone cells.

"Vicious cycle" between prostate cancer and bone stroma

Laboratory observations

While clinical human prostate cancer is predominantly osteoblastic, the established human prostate human cancer cell lines inoculated and grown in the bone of immune-compromised mice yield both osteoblastic and osteolytic lesions. Apparently, prostate cancer cells can participate in the process of bone turnover by exhibiting properties similar to osteoblasts, the so-called "osteomimetic" properties of prostate cancer cells as reported earlier (Koeneman et al., 1999). Much evidence supports this interesting phenotype of prostate cancer cells, in which they behave like osteoblasts. Prostate cancer cells express both soluble and membrane-bound RANK ligands and were shown to participate directly in osteoclastogenesis (Koeneman et al., 1999; Matsubara et al., 2001; Zhang et al., 2001; Yeung et al., 2002). Prostate cancer cells expressed a number of non-collagenous bone matrix proteins, such as osteocalcin, osteopontin, osteonectin and bone sialoprotein, alkaline phosphatase, and a key transcription factor, Runx 2 (cbfa1) that controls the transcription of osteocalcin and collagenous-3 (D'Alonzo et al., 2002). In addition, upon exposure to mineralizing cell culture conditions, prostate cancer cells have been shown to form *bona fide* mineralized bone crystals as detected by electron microscopy (Lin et al., 2001). These observations raise the possibility that soluble and/or matrix-associated molecules may be responsible for signaling between prostate cancer and bone stromal cells. Since bone-homing prostate cancer cells seek to adhere, colonize, and survive in bone, it is of pivotal importance to find out how prostate tumor and bone cells interact with the hope of identifying novel therapeutic targets for the treatment of prostate cancer bone metastasis. One attractive hypothesis is that prostate cancer cells may behave like osteoblasts and functionally participate in bone turnover. By markedly increasing the basal rate of bone turnover, this may further enhance prostate cancer cell colonization in bone (Cher, 2001; Nemeth et al., 2002). This hypothesis is supported by some clinical observations, where bisphosphonates, an effective class of agents that slow down or inhibit bone resorption, have been shown to reduce cancer cell colonization in experimental models of prostate and breast cancers (Coleman, 2001; Lee et al., 2001). In men harboring prostate cancer, there is evidence that increased bone resorption occurs upon castration. Whether these changes in bone turnover subsequent to hormonal manipulation or bisphosphonate treatment after prostate cancer cell colonization in bone affect the natural history of prostate cancer progression should be the subject of future thorough investigation.

Factors driving the "vicious cycle" between prostate cancer and bone cells

Guise and colleagues (Chirgwin and Guise, 2000) and Mundy (Mundy, 2002) presented the concept of a "vicious cycle" involving TGF-β produced by bone cells that promotes the production of PTHrP by many of tumor cells, including prostate and breast tumor cells. PTHrP stimulates bone turnover by enhancing osteolytic reaction in the bone. Increased release of TGF-β could result from rapid bone turnover, and this may trigger increased PTHrP production by cancer cells. The production of PTHrP by tumor cells will induce osteolytic cells to express an increased level of RANK ligands, which can promote osteoclast formation/activation and subsequently increased bone resorption. The enhanced resorptive process by osteoblasts and osteoclasts leads to "bone pitting" and subsequent colonization by cancer cells in the skeleton and associated bone destruction often observed in cancer patients. Thus, a "vicious cycle" may exist between TGF-β, PTHrP, RANK ligands in osteolytic prostate cancer. Interrupting the vicious cycle in cancer models using anti-PTHrP antibodies or osteoprogerin (OPG) has been shown to reduce colonization of cancer metastasis to bone (Zhang et al., 2001).

Transforming growth factor-β (TGF-β) is a 25-kDa disulfide-linked polypeptide which coordinates cell function over distances by binding to cell surface receptors. An immunohistochemical study of mouse prostate development indicated that TGF-β1 is expressed in mesenchymal cells (Timme et al., 1995). It was initially characterized by its effects on epithelial function and proliferation (Cui et al., 1995), but it is also an important mediator of stromal reaction (Wakefield and Roberts, 2002). Responses to TGF-β include phenotypic changes affecting adhesion, migration, differentiation, and cell fate. In general, TGF-β stimulates the production of ECM components, inhibits degradation, and alters integrin expression. It follows that all of these effects can significantly alter cell behavior.

TGF-β is abundant in latent forms that circulate or are bound to the ECM in bone. Activated TGF-β can bind to ubiquitous heterodimeric receptors and induce signal cascade through the SMAD pathway (Taipale et al., 1998). Stromal and epithelial cells of malignant and nonmalignant prostatic tumors express all three TGF-β isoforms and their related receptors which act as paracrine and autocrine factors, influencing prostate function and stromal-epithelial cell interaction (Cardillo et al., 2000). These data indicate that TGF-β1 produced by carcinoma cells acts on the surrounding stromal cells, which in turn induces stromal cells to release cytokines to further promote the malignancy of the cancer cells.

In addition to the TGF-β and PTHrP connection, a number of other candidate molecules may also contribute to the vicious cycle of cancer growth and bone meta-

stasis. For example, bone is a rich source of hydrogen peroxide, and hydrogen peroxide has been shown to increase the production of vascular endothelial growth factor (VEGF) by tumor cells. There is evidence that increased VEGF could further stimulate increased production of hydrogen peroxide by tumor and bone cells. Since VEGF is known to be required to support tumor growth and colonization, it is possible that a hydrogen peroxide/VEGF connection contributes to the vicious cycle between tumor and bone cells (Arbiser et al., 2002).

Endothelium-1 (ET-1) may also contribute to osteoblastic reaction when prostate cancer cells colonize to bone. ET-1 production is negatively regulated by androgen. Thus castration could potentially reduce osteoblastic reactions in bone through the reduction of ET-1. However, ET-1 and its interaction with receptor ET-1A could participate in the osteoblastic reaction and spur the vicious cycle in prostate cancer and bone by increased production of IL-1α, IL-1β, TNF-α, and TGF-β (Le Brun et al., 1999; Granchi et al., 2001). Increased production of TGF-β, EGF and IL-1β has been shown to upregulate ET-1, hence altering the growth factor and cytokine milieu in bone in response to ET-1 growth factor (Granchi et al., 2001). Cytokines may contribute to further prostate cancer growth and colonization to bone.

ET-1, composed of 21 amino acid residues, was originally isolated from porcine aortic endothelial cells (Kurihara et al., 1989). ET-1 is one of the four families of vasoactive peptides that include endothelin-2 (ET-2), endothelin-3 (ET-3), and endothelin-4 (ET-4) (Cunningham et al., 1997). All members of the endothelin family contain two essential disulfide bridges and six conserved amino acid residues at the C-terminus. In addition, they all are synthesized as pre-pro-polypeptides which need to be cleaved to produce pro-polypeptides. The pro-ET-1 is proteolytically cleaved by a membrane-bound metalloproteinase, endothelin-converting enzyme (ECE-1), produced by endothelial and epithelial cells (Xu et al., 1994). Two receptors for endothelins have been characterized, designated ETA and ETB. Although these receptors are structurally and functionally different, they share some similarities. Both are seven membrane domain receptors coupled through G proteins to phospholipase C. Both have an N-terminal signal sequence and a long N-terminal extracellular domain (Sakurai et al., 1992). ETA shows a higher affinity for ET-1 than for ET-2 and the lowest affinity for ET-3. The ETB receptor shows approximately equal affinity for each of the endothelins. Both ETA and ETB have been identified in prostate tissue. Stroma has higher concentration of ETA, while ETB is predominately in the epithelial cells of the prostate (Remuzzi and Benigni, 1993).

In human prostate cancer progression, ET-1 and ETA expression is retained, whereas ETB receptor expression is reduced. ET-1 protein expression was detected *in situ* in 14 of 14 primary cancers and 14 of 16 metastatic sites. Exogenous ET-1 induces prostate cancer proliferation directly and enhances the mitogenic effects of IGF I, IGF II, PDGF, bFGF, and EGF in serum-free conditions *in vitro*. ETA antagonist A-127722 inhibits ET-1-stimulated growth, but the ETB-selective receptor antagonist BQ-788 does not. ET-3, an ETB-selective agonist, also had no effect on prostate cancer growth. No specific ETB-binding sites could be demonstrated in any established human prostate cancer cell line tested, and ETB mRNA, detected by reverse transcription PCR, was reduced. The predominance of ETB binding in human benign prostatic epithelial tissue is not found in metastatic prostate cancer by autoradiography. Furthermore, a study of ET-1 in prostate cancer bone metastasis demonstrated that ET-1 is mitogenic for osteoblasts, inhibits osteoclastic bone-resorption, and induces the formation of osteoblastic lesions. All this suggests that ET-1 is involved in the new bone formation associated with prostate cancer metastasis (Nelson et al., 1999).

Tests of the mitogenic property of ET-1 indicated that other factors also can be co-factors with ET-1, such as bFGF and IGFs. PDGF and ET-1 also can play a role in tumor angiogenesis in conjunction with VEGF. Clinical trials of ET-A receptor antagonist in prostate cancer indicated that it could help patients, if they could tolerate mild but pervasive symptoms related to ET-1's vasoconstrictive effects (Kopetz et al., 2002).

A recent study (Taichman et al., 2002) showed that stromal chemokine and receptor, such as stromal cell-derived factor-1 (SDF-1 or CXCL12) and its receptor (CXCR4), may play a role as prostate cancer bone metastasis homing signals. The level of CXCR4 increased with the malignancy of the prostate cancer cell lines by both RT-PCR and Western blot analysis. The increased expression of CXCR4 also increased spreading to bone in animal studies. An *in vitro* study of cellular spreading in basement membrane indicates that spreading can be inhibited by CXCR4 antibody. These findings suggest that chemokine and its receptor could also be important in prostate cancer bone metastasis.

Together, these studies indicate that the process of prostate cancer bone metastasis is a complicated pathway requiring multiple chemokines, cytokines, and membrane proteins. These complexities also suggest the possibility of therapeutic strategies specifically focused on co-targeting and disrupting key carcinoma-stroma interactions.

Cancer therapy based upon co-targeting tumor and stroma

Laboratory and clinical observations

Because prostate cancer growth is highly susceptible to tumor-microenvironment interaction and experimen-

tally can be promoted by stromal fibroblasts, it is reasonable that control of prostate tumor growth might be optimized by co-targeting both tumor and stroma. To explore this concept, we designed studies to co-culture prostate cancer cells and bone stroma *in vitro*, establishing chimeric tumor models consisting of human prostate cancer cells and bone stroma. By introducing a "bystander" therapeutic gene, herpes simplex thymidine kinase (hsv-TK), to stromal cells only, we observed effective cell kill in tumor epithelium *in vitro* and shrinkage of tumor size *in vivo* upon addition of a pro-drug, gancyclovir (GCV). Since there were no identifiable gap junctions between prostate tumor cells and bone stroma under the electron microscope, we concluded that there must be metabolic cooperation between tumor epithelium and bone stroma mediated by soluble factors and extracellular matrices. By interrupting this communication, and targeting both tumor and stroma, tumor growth and survival may be adversely affected. Conceptually, co-targeting tumor and stroma in prostate cancer bone metastasis is a rational approach to the "vicious cycle" constantly operating between tumor and stroma. Directly inducing cell-kill of tumor epithelium and starving cancer cells by disrupting tumor interaction with the stromal compartment could achieve the best possible tumor regression.

In our laboratory, we co-targeted tumor and stroma using an adenoviral vector in which therapeutic gene expression was controlled by a tissue-specific and tumor restrictive promoter, such as osteocalcin, osteonectin, or bone sialoprotein. These have been shown to be highly effective in inducing long-term tumor regression, and even some cure in pre-established tumor in the skeleton with administration of the adenovirus through the intravenous route (Hsieh and Chung, 2001; Matsubara et al., 2001; Hsieh et al., 2002). This concept of bone targeting to improve therapeutic effects has received clinical support. Tu and colleagues (Tu et al., 2001) reported a significant prolongation of patient survival by targeting bone with strontium 89 and prostate tumors with chemotherapy.

Molecular basis of co-targeting

The bone microenvironment was depicted by Paget over a century ago as a specialized "soil" that favors the metastasis of certain selective cancer cell types ("seed"). While the precise mechanism by which cancer cells home to bone is still unknown, several attractive ideas and hypotheses have been proposed. Bone must express certain chemo-attractants that selectively retain circulating cancer cells, and cancer cells must express cognate ligands or receptors allowing them to attach to bone marrow-associated endothelial cells, marrow stromal cells or osteoblasts, and/or respond to bone-derived growth factors, cytokines/chemokines or extracellular matrices. To metastasize to bone, cancer cells must be able to survive "hostile" circulatory compartments, including the blood and lymphatic channels. The mere detection of cancer cells in blood or marrow stromal compartments may not reflect the "vitality" of cancer cells. Solakoglu, et al (Solakoglu et al., 2002) recently demonstrated that the outgrowth of cytokeratin-positive tumor cells from bone marrow can be detected in 81% of prostate cancer patients. Increased cell viability in patients correlated with increased cancer-related deaths.

From our use of prostate cancer cell lines as a model to study carcinoma-stroma interaction, we suggest that a switch of transcriptional factors must occur during the pathogenesis of prostate cancer. This biochemical switch could occur early, even when epithelial cells are still in the primary stage, since even then the expression of bone-like proteins such as osteocalcin, osteopontin, osteonectin, and bone sialoprotein was detected. Considering how osteocalcin promoter in prostate cancer cells is regulated, a vicious cycle could occur at the level of transcription factor activation, wherein the coordinated activation of transcription factors by known soluble factors and ECM-integrin signaling culminates in the ability of prostate cancer cells to proliferate and survive in bone. Numerous links have been established between the up-regulation of transcription factors such as Runx-2 and the potential alteration of cellular behavior that could lead to increased cell growth and spread to bone.

Runx-2 is a potent and specific transcription factor that controls mesenchymal-epithelial interaction in tooth development (D'Souza et al., 1999). Apparently, Runx-2 activation is controlled by soluble growth factors, and upon activation it can regulate soluble growth factor secretion, which ultimately controls the growth and differentiation of enamel tooth epithelium. Based upon this and other published data, we proposed that activation of similar transcription factors such as Runx-2 in prostate cancer cells could potentially enhance prostate cancer cell invasion and migration through the induction of collagenase (e.g., collagenase 3) and other metalloproteinases. The concomitant induction of Runx-2, collagenase 3, and other growth and differentiation supportive factors could enhance prostate cancer survival and invasion. Similarly, the activation of the $\alpha V\beta 3$ and $\alpha 2V\beta 1$ integrin-ECM pathways may promote outside-in signals that result in enhanced cell migration and invasion.

We proposed earlier that prostate cancer metastasis to bone is not a random process. It involves the specific recognition of cancer cells by bone as "self" and the production of bone-like proteins by cancer cells. The expression of bone-like proteins by prostate cancer cells may allow them to adhere, proliferate and survive in the bone microenvironment and participate in certain normal functions of bone cells, i.e., bone resorption.

Cancer cells express a bone-like phenotype early, when they are in primary lesions. This raises the possi-

bility that the expression of bone-like proteins by cancer cells and reactive stroma may serve as a prognostic biomarker for prostate cancer bone metastasis and possibly as a predictor for patient survival. By combining the expression of bone-like proteins and the stromal reaction to epithelium, it is possible that novel molecular markers can be developed both at the gene expression and genetic level. While the role of bone-like protein is presently unclear, it is possible that the activation of these processes may occur at the transcription level. Transcriptional factor switching could be of fundamental importance in determining the phenotype of cancer cells and might influence the extent of the vicious cycle between tumor cells and bone stroma. It is possible that specific targeting of transcriptional factors could have benefit as cancer therapy. Interrupting the activation of bone-like proteins in tumor epithelium and bone stroma may prevent prostate cancer cell adherence, proliferation, and survival in bone.

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